EMBO reports

Manuscript EMBOR-2011-35415

The FIH hydroxylase is a cellular peroxide sensor that modulates HIF transcriptional activity

Norma Masson, Rachelle S. Singleton, Rok Sekirnik, David C. Trudgian, Lucy J. Ambrose, Melroy X. Miranda, Ya-Min Tian, Benedikt M. Kessler, Christopher J. Schofield and Peter J. Ratcliffe

Corresponding author: Norma Masson, Oxford University

Review timeline:

Submission date:	16 September 2011
Editorial Decision:	06 October 2011
Revision received:	08 December 2011
Editorial Decision:	22 December 2011
Revision received:	28 December 2011
Accepted:	09 January 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 06 October 2011

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, all the referees find the topic of interest and in principle suitable for us. Nevertheless, referees 1 and 2 in particular point out several important issues that should be addressed before publication can be considered. Referee 3 also requests an additional experiment.

In this case, taking the scope of EMBO reports into account, it would be particularly important to demonstrate the physiological relevance of the findings (regarding endogenous levels of peroxide in cells), prove the direct link between peroxide level and HIF status, and reconcile your results with previous studies, as requested by referees 2 and 3. Please address also the other concerns of the referees, as doing so would strengthen the study.

Given the constructive suggestions provided by the referees on how to improve your study, I would like to give you the opportunity to revise your manuscript. If the referee concerns can be adequately addressed, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In this interesting manuscript, Masson et al. have investigated possible peroxide sensing functions of HIF hydroxylases. The basis of this study is that a significant controversy exists in the published literature as to whether reactive oxygen species such as peroxide play a role in cellular oxygen sensing in the HIF pathway through the inactivation of HIF hydroxylases. The current manuscript provides compelling evidence that the FIH asparaginyl hydroxylase is significantly more sensitive to cellular peroxide levels than prolyl hydroxylases (PHDs) which indicates that while peroxide levels may be modulatory, they are unlikely to be primarily involved in oxygen sensing via the HIF pathway. While this is a well carried out study with clear and important conclusions, some addition experiments would strengthen the impact of the manuscript.

Major Issues:
1) A key question which pertains to the physiologic importance of the observations reported in the context of hypoxia is: how do the levels of intracellular peroxide experienced by cells following the addition of T-hydro compare to endogenous peroxide levels seen in basal or stimulated conditions. In other words, does the T-hydro treatment accurately reflect the oxidant stress experienced by cells during hypoxia (or some other co-stimulus such as LPS). To address this, the authors should measure the redox status of the peroxide treated cells by for example measuring the reduction of glutathione or using a peroxide sensitive marker and compare this to the redox status of cells exposed to hypoxia or LPS. This will clarify whether changes in redox stress associated with hypoxia impact upon HIF signaling via FIH or whether the results pertain more to situations where the source of the redox stress is independent of hypoxia (e.g. inflammatory activity).

2) It would be interesting to monitor activation of the well characterized redox-responsive transcription factor NRF2 as a surrogate readout of cellular redox-sensing and determine if the cells are sensing peroxide levels in the physiologic range. For example, if NRF2 is activated under conditions where HIF is not, this would strengthen the argument that redox status and HIF activation are not primarily coupled.

3) Can the effects of ter-butyl hydroperoxide on FIH activity be overcome by pre-treatment with antioxidants?

4) In Figure 5A, normoxic controls should be included to demonstrate decreased P402/P564/N803 hydroxylation with hypoxia alone (in cells not treated with ter-butyl hydroperoxide).

Minor Issues:

1) In the abstract the authors state that "Hypoxic and oxidant stress co-exist in biological systems". The literature relating to the induction of oxidative stress in hypoxia is controversial. A number of studies also indicate that reactive oxygen species may be decreased in hypoxia. This controversy should be acknowledged by citation of the relevant literature.

2) In figure 2, some of the bars have error bars while some do not. The reason for this should be explained.

3) In the results section "Peroxide rapidly inhibits FIH in a range of cell types" a number of

acronyms need to be spelt out (e.g. WGE, RRL, IB).

Referee #2:

Masson and colleagues show that the factor inhibiting HIF (FIH), a well-established asparaginyl hydroxylase, exhibits much greater sensitivity towards peroxide as compared to PHDs, which are prolyl hydroxylases that also target HIF and respond to peroxide treatment. Using U2OS osteosarcoma cells, RCC4 renal carcinoma cells as well as in vitro systems, the authors found that exposure to ROS or peroxide rapidly inhibits FIH activity, probably by direct modification on FIH, which then leads to a corresponding change of HIF asparaginyl hydroxylation and transcriptional activity. Although the molecular mechanism underlying how exactly FIH is modified in response to peroxide was uncharacterized, the fact that low oxygen and oxidant stress could regulate HIF in a mutually-independent fashion is intriguing and should be appreciated by researchers in the hypoxia field. However, the authors must address the important point that in previous studies, oxidant stress clearly affected HIF subunit accumulation which must depend on PHD activity. How can these findings be reconciled with multiple previous reports (Chandel, Mansfield, Pan, etc.)?

The following additional concerns should be addressed:

1. The blots of total FIH protein need to be shown in Figure 1 and 2 to confirm that the changes of asparaginyl hydroxylation were not due to a change in FIH expression. This is particularly the case in Figure 1C, where the authors argued that 10uM T-hydro affected HIF expression in RCC4 cells.

2. It's useful to know whether asparagine 851 within HIF-2α is also more sensitive to peroxide than proline hydroxylation by PHD. RCC4 cells express both HIF-1α and HIF-2α.

3. In Figure 2, the error bars of column 1, 2, 5, 6, 7, 8 are missing.

4. In Figure 3E, knocking-down expression or inhibiting activity of NAPDH oxidase is recommended to see if it can rescue the effect of LPS on THP-1 cells.

5. Although QPCR has been performed on a few HIF-1α target genes in Figure 5C, at least one luciferase assay using the HRE (hypoxia response element) reporter, as well as the immunoprecipitation of HIF-1α and CBP/p300 are necessary to prove the direct link between peroxide treatment and HIF status.

Referee #3:

This is an interesting and potentially important paper for the field of oxygen sensing. Ratcliffe and colleagues have performed rigorous experiment to conclude that FIH is a peroxide sensor. This finding has huge implications beyond oxygen sensing. FIH is also likely a major metabolic regulator. Diseases such as obesity are characterized by high levels of oxidative stress thus might alter FIH activity. Thus the paper has broad implications.

The paper is suitable for EMBO reports. However, I have one set of experiments that would be of great interest to the oxygen sensing community.

Multiple laboratories have utilized mitochondrial targeted antioxidants as tool to prevent ROS production during hypoxia. These antioxidants prevent hypoxic stabilization of HIF-1alpha protein.
(1) Patten et al. Mol Biol Cell. 2010 Sep 15;21(18):3247-57.
(2) Bell et al. Journal of Cell Biology J Cell Biol. 2007 Jun 18;177(6):1029-36.
(3) Sanjuán-Pla A, FEBS Lett. 2005 May 9;579(12):2669-74.
(4) Wang D, Malo D, Hekimi S. J Immunol. 2010 Jan 15;184(2):582-90.

The investigators should analyze the hydroxylation status of proline and asparagine residues under hypoxia (1-2% O2) in the presence of a mitochondrial targeted antioxidant.

1st Revision - authors' response 08 December 2011

Response to Referees:

Referee #1:

In this interesting manuscript, Masson et al. have investigated possible peroxide sensing functions of HIF hydroxylases. The basis of this study is that a significant controversy exists in the published literature as to whether reactive oxygen species such as peroxide play a role in cellular oxygen sensing in the HIF pathway through the inactivation of HIF hydroxylases. The current manuscript provides compelling evidence that the FIH asparaginyl hydroxylase is significantly more sensitive to cellular peroxide levels than prolyl hydroxylases (PHDs) which indicates that while peroxide levels may be modulatory, they are unlikely to be primarily involved in oxygen sensing via the HIF pathway. While this is a well carried out study with clear and important conclusions, some addition experiments would strengthen the impact of the manuscript.

Major Issues:

1) A key question which pertains to the physiologic importance of the observations reported in the context of hypoxia is: how do the levels of intracellular peroxide experienced by cells following the addition of T-hydro compare to endogenous peroxide levels seen in basal or stimulated conditions. In other words, does the T-hydro treatment accurately reflect the oxidant stress experienced by cells during hypoxia (or some other co-stimulus such as LPS). To address this, the authors should measure the redox status of the peroxide treated cells by for example measuring the reduction of glutathione or using a peroxide sensitive marker and compare this to the redox status of cells exposed to hypoxia or LPS. This will clarify whether changes in redox stress associated with hypoxia impact upon HIF signaling via FIH or whether the results pertain more to situations where the source of the redox stress is independent of hypoxia (e.g. inflammatory activity).

The reviewer asks whether T-hydro treatment reflects the oxidant stress experienced by cells in hypoxia and suggests that we might address this by making measurements using a peroxide sensitive marker in the two conditions. As indicated in the reviewer's

summary paragraph, this question has been raised because of the controversy regarding whether production of reactive oxygen species (ROS) and oxidant stresses, which are reported (by some) to be increased in hypoxia, are responsible for the reduction in HIF hydroxylase activity that is observed in hypoxia.

As the referee points out later (minor point 1) this is now the subject of a very large and conflicting literature, in which some authors have reported increased, and some have reported reduced, levels of reactive oxygen species (ROS) in hypoxia. Some of this work has related these measurements to changes in HIF levels and (in some cases) to changes in HIF hydroxylase activity (though most studies have not measured this directly). Many opinions have been given as to why these measures of ROS or oxidant stresses have yielded different results. These include, uncertainty about what is actually being measured, whether the ROS probes alter the production of ROS themselves and whether the measurement methods have the necessary chemical specificity and time/spatial resolution to properly reflect actions that are hypothesized to take place on the HIF hydroxylase enzymes (reviewed in part in, Cash *et al*. Free Radic Biol Med 43(9) 1219 (2007), Hoffman *et al*. Am J Physiol Heart Circ Physiol. 2007 Jan; 292(1): H101-8). Given all the difficulties that have been generated by this approach, including the uncertainty about what is actually being measured, we do not think that it would be appropriate to attempt to resolve this controversy by further measurements using the same or similar methods in the context of this manuscript, if indeed this is possible at all.

In part, it was to address this very refractory problem that we have taken the approach we have i.e. to measure changes in the hydroxylation status of specific amino acids in HIF-1alpha and changes in the activity of specific hydroxylase enzymes to a defined agent (T-hydro and hydrogen peroxide itself) and relate the effects to known effects of hypoxia on these enzymes - particular quantified responses to graded hypoxia that were previously established in this laboratory (Tian et al. 2011 J Biol. Chem. 286 13041-13051). We consider that the diametric contrast we observed between established sensitivity to hypoxia and our analysis of sensitivity to peroxide for different enzymes is strong evidence for fundamentally different mechanism of inhibition by hypoxia and peroxide. This is important because sensitivity of HIF hydroxylation to peroxide (frequently inferred and sometimes measured) has been

used as a key argument for the hypothesis that oxidant stress is the regulator of enzyme activity in hypoxia. We consider that the data provided in this manuscript provides important evidence that this is unlikely to be the case (and in their overall assessment of the work, the three referees appear to agree).

We do however accept the reviewer's point (this comment and point 2, below) that measuring the effects of hypoxia and T-hydro/hydrogen peroxide (in exactly the way we have used them) on an endogenous physiological response to oxidant stress (i.e. the well characterized redox-responsive transcription factor NRF2) would be of additional interest and in response to this suggestion we have made measurements of NRF2 under the relevant conditions (see below).

2) It would be interesting to monitor activation of the well characterized redox-responsive transcription factor NRF2 as a surrogate readout of cellular redox-sensing and determine if the cells are sensing peroxide levels in the physiologic range. For example, if NRF2 is activated under conditions where HIF is not, this would strengthen the argument that redox status and HIF activation are not primarily coupled.

This is an interesting suggestion. We have monitored activation of NRF2 by immunoblotting of total NRF2 protein levels. We have used 3 NRF2 antibodies: Abcam ab31163 rabbit polyclonal anti-NRF2 (against amino acids 569-588 of human NRF2, R&D clone 383727 mouse monoclonal (raised against recombinant human NRF2 amino acids 17-605) and Novus Biologicals NBP1-36229 (also raised against amino acids 569-588 of human NRF2). With all 3 we detect a ~110 kDa NRF2 immunoreactive species (similarly described by others e.g. Lee *et al*. BBRC 2001; 280: 286-292, Singh *et al*. PLoS Med. 2006 Oct; 3(10):e420, Piccirillo *et al*. J Biol Chem. 2009 Oct 2; 284(40):27721-33), that is induced by T-hydro and (consistent with proteasomal regulation of NRF-2), by the proteasomal inhibitor MG132. We measured changes in NRF2 and HIF-1alpha levels in response to both graded hypoxia and increasing doses of T-hydro. The results demonstrate that under conditions in which hypoxia induces robust up-regulation of HIF-1alpha (implying suppression of HIF prolyl hydroxylation), there was little or no induction of NRF2 (revised manuscript, supplementary figure 7A). In contrast, whilst T-hydro (as expected)

induced NRF2 it did so at low doses (5-10mM) which, whilst sufficient to inhibit FIH, were not sufficient to induce HIF protein (revised manuscript, supplementary figure 7B) or inhibit HIF prolyl hydroxylase activity (figure 3). This would suggest that if ROS levels are increased in hypoxia, they are not being sensed by NRF2. As the reviewer has indicated, this does indeed strengthen the argument that cellular redox status and HIF activation are not primarily coupled. The new experimental method and data has been added to the revised manuscript and the implications are discussed briefly in line with the referee's comment.

3) Can the effects of ter-butyl hydroperoxide on FIH activity be overcome by pre-treatment with antioxidants?

We agree that this is a relevant question. To address the point with have tested the effect of pre-treatment with Ascorbate (30mM) or N-acetyl cysteine (5mM) - two compounds that can potentially exhibit different types of antioxidant action. Neither type of pre-treatment overcame inhibition of FIH by peroxide. Note that (as expected) ascorbate did reduce total HIF-1alpha levels (supplementary figure 5A right panel) suggesting an action to promote HIF prolyl hydroxylation in normoxic cells (as reported by our laboratory, Knowles *et al*. Cancer Res. 2003 Apr 15; 63 (8):1764-8 and others). Thus, inhibition of FIH by peroxide appears to be relatively resistant to antioxidants. We have added the new data to the revised in supplementary Figure 5B and discussed this finding briefly. We would however like to add the caveat that since the intracellular chemistry of these agents is complex our results do not preclude some protection being observed under other circumstances.

4) In Figure 5A, normoxic controls should be included to demonstrate decreased P402/P564/N803 hydroxylation with hypoxia alone (in cells not treated with ter-butyl hydroperoxide).

In fact, in RCC4 cells cultured at 1% hypoxia, steady-state levels of hydroxylation are high at all three sites (ca 80% at N803 and ca 100% at P402 and P564). This is likely to be due to the relatively long half-life of HIF-1alpha in these VHL-defective cells, allowing hydroxylation to run almost to completion. We have worked extensively on this and published relevant datasets in Tian et al (2011) J Biol. Chem 286 13041-

13051. In the interests of space we have not duplicated this data, but to clarify the position we have referred to this work (indicating that we used conditions under which P402/P564/N803 hydroxylation would not be predicted to be much reduced by hypoxia alone) at the relevant point in the revised manuscript.

Minor Issues:

1) In the abstract the authors state that "Hypoxic and oxidant stress co-exist in biological systems". The literature relating to the induction of oxidative stress in hypoxia is controversial. A number of studies also indicate that reactive oxygen species may be decreased in hypoxia. This controversy should be acknowledged by citation of the relevant literature.

Yes, we are aware of this controversy (see above) and agree with the reviewer. In this context we have revised the manuscript to include specific references to studies that have reported decreases in reactive oxygen species in hypoxia as well as those that have reported increases.

2) In figure 2, some of the bars have error bars while some do not. The reason for this should be explained.

Thank you. This is because, although several replicate experiments were performed, gaining highly quantitative data from mass spectrometry depends on the quality of peptide separation by HPLC and on the ionization characteristics of the peptide and this can vary from experiment to experiment. In this series of experiments, robust quantitative data was obtained for the peptide containing Rabankyrin-5 N485 in every experiment (n=4), whereas this was not the case for all sites. This is because this peptide has particularly good characteristics for quantitation in our MS protocols (Singleton et al. (2011) J. Biol. Chem. 286 33784-33794). Nevertheless, reasonably good quantitative data was obtained at the other sites in some of the experiments and hence this data is also provided (but simply as a mean of two or a single measurement, hence no statistical analysis). This data adds to the experimental result since it demonstrates, across a range of sites, that whenever hydroxylation could be accurately quantified, FIH-dependent hydroxylation was severely reduced by peroxide. We apologize for not making this clear and revised the manuscript accordingly.

3) In the results section "Peroxide rapidly inhibits FIH in a range of cell types" a number of acronyms need to be spelt out (e.g. WGE, RRL, IB).

Thank you. We have altered the text to spell out these acronyms.

Referee #2:

Masson and colleagues show that the factor inhibiting HIF (FIH), a well-established asparaginyl hydroxylase, exhibits much greater sensitivity towards peroxide as compared to PHDs, which are prolyl hydroxylases that also target HIF and respond to peroxide treatment. Using U2OS osteosarcoma cells, RCC4 renal carcinoma cells as well as in vitro systems, the authors found that exposure to ROS or peroxide rapidly inhibits FIH activity, probably by direct modification on FIH, which then leads to a corresponding change of HIF asparaginyl hydroxylation and transcriptional activity. Although the molecular mechanism underlying how exactly FIH is modified in response to peroxide was uncharacterized, the fact that low oxygen and oxidant stress could regulate HIF in a mutually-independent fashion is intriguing and should be appreciated by researchers in the hypoxia field. However, the authors must address the important point that in previous studies, oxidant stress clearly affected HIF subunit accumulation which must depend on PHD activity. How can these findings be reconciled with multiple previous reports (Chandel, Mansfield, Pan, etc.)?

The reviewer asks us to address the finding that in previous studies oxidant stress affected HIF subunit accumulation and suggests that this must depend on PHD activity. We agree that this is an important point for discussion, but had necessarily limited discussion because of space constraints. There are several points to make.

First, we wish to emphasise that the point of our work is to directly compare the responses the two different HIF hydroxylase systems to a given set of conditions. Whilst we have attempted to follow published protocols (and give an exact description of our own) there could be differences in the total oxidant load. For instance, applying 10mM T-hydro in a medium volume of 1ml versus 10ml will likely affect the oxidant load, but these details are rarely given. It is therefore possible that (apparently) minor differences in these technical details have resulted in differences between our work and that of others (and indeed between different pieces of work published by others)

in terms of absolute sensitivity of HIF hydroxylase to peroxide (see also Schroder and Eaton. Curr. Opin. Pharm (2008) 8: 153-159). We should also clarify that at the higher exposures to T-hydro we did see effects on HIF levels (see original supplementary figure 1B), but these were small in comparison with those we observed in response to hypoxia or HIF prolyl hydroxylase inhibitors such as DFO. At even higher doses of T-hydro we observed more HIF accumulation; however these levels of peroxide likely have other relevant effects (see below).

Second, it is not actually clear that HIF subunit accumulation must depend on (changed) PHD activity, as many other factors affect the level of HIF sub-units and even when the steady-state level of HIF hydroxylation is measured factors other than hydroxylase activity (e.g. rate of HIF substrate production) may affect this steady-state level. We have ourselves studied this in some detail as a prelude to this work. For instance, it is established that oxidant stress both inhibits protein phosphatase activity and activates kinase activity (Paulsen and Carroll (2010) ACS Chem Biol. Jan 15; 5(1):47-62) therefore may alter the steady-state levels of phosphorylation (and activity) in growth signaling pathways. In this context we have noted that peroxide will activate the PI3K/Akt pathway a pathway that is known to promote translation of HIF-1alpha (Zhou *et al*. Cancer Res. 2004 64: 9041-48, Page *et al*. J.Biol. Chem. 2002 277: 48403-9, Laughner *et al*. Mol. Cell. Biol. 2001 21; 3995-4004) and hence the rate of production of the hydroxylase substrate sequences.

Third, most studies of HIF induction have used higher doses of peroxide. For instance, Chandel et al. (2000) J. Biol. Chem. 275 25130-25138 specifically state that hydrogen peroxide failed to stabilize HIF-1a when used at 10mM (and proceeded to use 25-40mM). We are concerned that higher doses of peroxide used in some studies could have toxic actions related to its pleiotrophic actions on intracellular biochemistry.
This is why we have focused our analysis of comparative studies and have performed both measures of steady-state hydroxylation and direct assays of enzyme activity. In the revised manuscript we have however added further data using higher doses of peroxide (which do induce HIF-1a) in supplementary figure S1 and figure 5D and referred this to the previous work.

The following additional concerns should be addressed:

1. The blots of total FIH protein need to be shown in Figure 1 and 2 to confirm that the changes of asparaginyl hydroxylation were not due to a change in FIH expression. This is particularly the case in Figure 1C, where the authors argued that 10uM T-hydro affected HIF expression in RCC4 cells.

Thank you. We have now included the requested FIH immunoblot data for the RCC4 cell experiment in Fig. 1C (and also for Fig. 5A). For the HEK 293 cells expressing the Rabankyrin-5/HIF-1alpha CAD fusion used in Figure 2, we have inserted the requested FIH immunoblot as a new supplementary Figure 2B.

2. It's useful to know whether asparagine 851 within HIF-2α; is also more sensitive to peroxide than proline hydroxylation by PHD. RCC4 cells express both HIF-1α and HIF-2α.

We agree, and in respect of this the transfected HIF-1a and HIF-2a polypeptides analysed in figure 1D, our data suggests that this is indeed the case. The hydroxy-residue-specific antibodies are raised against HIF-1a and their cross-reactivity with HIF-2a is very weak (see Tian et al. (2011) J. Biol. Chem. 286 13041-51). The analysis shown in figure 1D is therefore dependent on the existence of elevated levels of polypeptides. Though the referee is correct that RCC4 cells express both HIF-1a and HIF-2a, the available antibodies do not have the sensitivity to detect hydroxylated HIF-2 epitopes at endogenous levels. Nevertheless, the data in figure 1D does suggest that HIF-2a N851 is sensitve to peroxide. We have clarified this in the revised manuscript.

3. In Figure 2, the error bars of column 1, 2, 5, 6, 7, 8 are missing.

Thank you. In fact error bars were only applied to columns 3 and 4 because quantitative data for all replicates was restricted to this site. As indicated in the response to referee 1, although several replicate experiments were performed, gaining highly quantitative data from mass spectrometry depends on the quality of HPLC separation and on the ionization characteristics of the peptide which may vary from experiment to experiment. In this series of experiments, robust quantitative data was

obtained for the peptide containing Rabankyrin-5 N485 in every experiment (n=4), whereas this was not the case for all sites. This is because this peptide has particularly good characteristics for quantitation in our MS protocols (Singleton et al. (20110 J. Biol. Chem. 286 33784-33794). Nevertheless reasonably good quantitative data was obtained at the other sites in some of the experiments and hence this data is also provided (but simply as a mean of two or single measurement, hence no statistical analysis). This data adds to the experimental result since it demonstrates, across a range of sites, that whenever hydroxylation could be accurately quantified, FIH-dependent hydroxylation was severely reduced by peroxide. We apologize for not making this clear and have revised manuscript to explain the analysis more precisely.

4. In Figure 3E, knocking-down expression or inhibiting activity of NAPDH oxidase is recommended to see if it can rescue the effect of LPS on THP-1 cells.

We agree that this is an interesting question, though it is not straightforward to resolve. Whilst it is implied that the observed inhibition of FIH is due the activity of NADPH oxidase (or related enzymes) this is not proven. Unfortunately neither chemical nor genetic inhibition of these enzymes is completely straightforward. Diphenylene iodonium (DPI) and related compounds have been used as inhibitors of these enzymes but are not specific, since they inhibit other flavoproteins, including components of the electron chain. From the genetic perspective, multiple NADPH oxidase (NOX) isoforms exist. Optimizing then assaying and controlling the individual/combined interventions with siRNA/shRNA, would be necessary to properly address this issue. This would be a considerable undertaking and not feasible within the time-limits allowed for revision, or readily described within the space allowance for this type of report. We therefore think that such an analysis would better form part of another study. However we accept the referee's point and (since we are also modifying the manuscript to provide extensive new data within the allowed format) we would therefore prefer to remove this dataset entirely pending definitive analysis.

5. Although QPCR has been performed on a few HIF-1α target genes in Figure 5C, at least one luciferase assay using the HRE (hypoxia response element) reporter, as well

as the immunoprecipitation of HIF-1α and CBP/p300 are necessary to prove the direct link between peroxide treatment and HIF status.

Whilst we accept the referee's point that more mechanistic dissection could be of interest, all the genes assayed are proven direct transcriptional targets of HIF-1alpha, none are known (or shown, figure 5C) to be inducible by cellular stress alone, effects of peroxide are dependent on FIH and our analysis clearly proves effects of peroxide on the asparaginyl hydroxylation of HIF-1alpha. On this basis we do not think that the implication that we have not proven a direct link between HIF status and peroxide treatment (if that is what is intended by the comment) is entirely fair.

Nevertheless we have performed further experiments using luciferase assays and HRE reporters as requested. To be consistent with our other work, these experiments were performed over a 4 hour time-course. The results show that, concurrent with reduction in HIF-1alpha asparaginyl hydroxylation, in peroxide treated cells, there is indeed an HRE-dependent increase in transcriptional activation of the reporter gene. Note that at this early time point (chosen for consistency with other aspects of our work, but unusual for reporter gene measures of HIF activation) the effect of peroxide is more marked than hypoxia - suggesting that the time course of different modes of activation might be different. Overall, the new data adds to the evidence that peroxide-dependent suppression of HIF asparaginyl hydroxylation can contribute to activation of HIF/HRE dependent transcription and in response to the referee's request we have added it into the revised manuscript (supplementary figure 6). Published work using in vitro analyses and structural predictions have indicated HIF-1alpha asparginyl hydroxylation blocks recruitment of p300/CBP, though whether these effects account for all observed effects of FIH on HIF/HRE-dependent remains an open question. Whilst this is an interesting (and rather substantial) field of work in its own right - it is somewhat separate from the main focus of this paper (on differential sensitive of HIF prolyl and asparaginyl hydroxylation to peroxide). In the interests of timely resubmission and meeting space limitations, we have therefore restricted ourselves to performing the reporter gene assays as requested, but have had a caveat in respect of the referee's point in the revised manuscript.

Referee #3:

This is an interesting and potentially important paper for the field of oxygen sensing. Ratcliffe and colleagues have performed rigorous experiment to conclude that FIH is a peroxide sensor. This finding has huge implications beyond oxygen sensing. FIH is also likely a major metabolic regulator. Diseases such as obesity are characterized by high levels of oxidative stress thus might alter FIH activity. Thus the paper has broad implications.

The paper is suitable for EMBO reports. However, I have one set of experiments that would be of great interest to the oxygen sensing community.

Multiple laboratories have utilized mitochondrial targeted antioxidants as tool to prevent ROS production during hypoxia. These antioxidants prevent hypoxic stabilization of HIF-1alpha protein.

(1) Patten et al. Mol Biol Cell. 2010 Sep 15;21(18):3247-57.

(2) Bell et al. Journal of Cell Biology J Cell Biol. 2007 Jun 18;177(6):1029-36.

(3) Sanjuan-Pla A, FEBS Lett. 2005 May 9;579(12):2669-74.

(4) Wang D, Malo D, Hekimi S. J Immunol. 2010 Jan 15;184(2):582-90.

The investigators should analyze the hydroxylation status of proline and asparagine residues under hypoxia (1-2% O2) in the presence of a mitochondrial targeted antioxidant.

We appreciate the reviewer's support for our work. Whilst we accept that defining the action of mitochondrially targeted antioxidants on the oxygen-sensitivity of these enzymes could be of interest, proper analysis of such actions (to define if there is indeed an action on HIF hydroxylase activity itself) would entrain a very considerable amount of work and is a substantially separate issue. We therefore consider that any such analyses would be the subject of a different study. For instance, in relation to the reviewer's suggestion that we examine prolyl and asparaginyl hydroxylation in cells under 1-2% oxygen, we should point out to do this in cells in not absolutely straightforward. To permit comparison of prolyl and asparaginyl hydroxylation we have used VHL-defective cells (or proteasomally blocked cells) to prevent differential degradation of prolyl hydroxylated HIF-1alpha. Under these conditions, the half-life

of HIF-1alpha is prolonged and prolyl hydroxylation generally runs to completion under these conditions (Tian et al. (2011) J.Biol. Chem. 286 p13041-51); since the proposed action of mitochondrially targeted oxidants would be to increase hydroxylase activity, it would not be possible to measure such an increase in this way.

2nd Editorial Decision 22 December 2011

Thank you for your patience while we have peer-reviewed your revised manuscript. We have now received the enclosed reports on it. Although all referees acknowledge the work performed during revision, referees 2 and 3 consider that it has not been conclusively shown that oxidant stress is not signaling to PHDs.

Although referee 1 is satisfied in this respect, it does seem appropriate to tone down the claims or perform the requested experiment (analysis of hydroxylation in the presence or absence of antioxidants). I feel that an extended discussion of this issue would suffice in this case.

Browsing through the manuscript, I have noticed that the legend to figure 5C is not complete with respect to statistical information. The identity of the values represented (mean?) and of the error bars is not stated.

I look forward to seeing a new revised version of your manuscript as soon as possible.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors have largely addressed my comments regarding the manuscript and I am now happy to recommend it for publication in EMBO reports.

Referee #2:

The revised manuscript by Masson and colleagues contains improvements over the previous version. One of the key added-on results, which strengthens the paper, is that cells treated with low concentration of T-hydro exhibit clear activation of redox-sensing transcriptional factor NRF2 without changing the expression level of the HIFα protein. This fact indicates that HIF proline-hydroxylases (PHDs) are not sensitive to the particular oxidant stress applied in this study, while NRF2 and FIH both respond. However, this result is not sufficient to support the authors' argument that cellular redox status and HIF activation are primarily uncoupled, given the technical difficulty to further examine the oxidative status of T-hydro-challenged cells. Under some physiological circumstances, cells can certainly use ROS as a regulator of PHD activity. For example, Gerald et al. (2004) have shown that JunD-null MEFs exhibit normoxic HIF stabilization due to lower levels of ROS-scavenging enzymes, including glutathione-S-transferase-1 and cysteine dioxygenase. Moreover, a number of other researchers, including Chandel, Mansfield, Pan, etc. have reported that exogenous H2O2 increases HIF stability in different cellular settings. Although they often use higher doses of peroxide to inhibit PHD activity, the increased stability or other unique features of T-hydro make it difficult to compare the physiological effects of various peroxide reagents. Taken together, the authors should make changes in their wording to weaken the conclusion that oxidant

stress is not signaling directly to PHDs in vivo, or that hypoxia does not include oxidant stress. Based on the results of the current paper, it is impossible to conclude that hypoxia does not include intracellular changes in ROS. Importantly, hypoxia must be applied in the presence or absence of anti-oxidants using intact cells (which are pVHL replete) to make this claim. Finally, in my opinion, the authors have not adequately responded to criticisms raised by Reviewers #1 and #3.

Referee #3:

This revised version has attempted to address all the concerns the referees raised. The paper is experimentally well done and the data justifies the claim that FIH is more sensitive than PHDs to peroxide. However, the authors cannot conclude anything about hypoxia and ROS. Hydroxylation should be analyzed with or without antioxidants in order to reach the conclusions stated in the study.

I nevertheless think the paper is worth publishing because it will generate lots of discussion and will be thought provoking. I believe these things sort themselves out over time.

However, the authors should refrain from making any conclusions about how FIH or PHDs respond to hypoxia.

2nd Revision - authors' response 28 December 2011

Please find attached our revised manuscript.
As suggested we have revised the manuscript in discussion to avoid over-interpretation of what we are claiming. We note that some of the referees' concerns appear to relate to the dialogue with referee 1 rather than statements we make in the manuscript itself.

In particular we are not claiming that 'hypoxia does not include oxidant stress' (as we have argued, this is not straight forward to measure). We have revised the discussion (paragraph 3) to make this explicit. We have also revised the discussion to make it explicit that our data do not exclude a role for 'oxidant stresses in signalling to PHD' (i.e. modulating activity).

As requested we have also made changes to the wording to reduce the risk of overstatement and to be as precise as possible in drawing out what we consider to be the significant implications of our work on the differential sensitivity of PHDs and FIH to peroxide, for theories on the regulation of these enzymes.

The legend to figure 5C has been modified and the references re-formatted as requested.

We hope that you will find the revised manuscript suitable for publication in EMBO Reports.

Thank you for your consideration.

3rd Editorial Decision 09 January 2012

Thank you for submitting the revised version of your study. I have now had time to assess it and I believe it satisfactorily addresses the remaining referee concerns. I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,
Editor
EMBO Reports

